Antigen Assay for
Rat Fibrinogen

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INTRODUCTION

Fibrinogen is a soluble glycoprotein that circulates in the blood and is converted to insoluble fibrin by thrombin in the final step of the coagulation cascade[1]. Hepatic expression of fibrinogen increases two to four hundred fold during the acute phase response to infection or inflammation[2]. Elevated fibrinogen levels are correlated with cardiovascular disease[3] and atherosclerosis[4].

PRINCIPLES OF PROCEDURE

This is an ELISA (Enzyme-Linked Immunosorbent Assay) for the quantitative analysis of total fibrinogen levels in rat plasma and serum. This test kit operates on the basis of sandwich ELISA where the fibrinogen binds to a capture antibody on the plate and is quantified with the use of a biotin labeled primary antibody and an avidin-HRP conjugate.

Rat fibrinogen will bind to the affinity purified capture antibody coated on the microtiter plate. After appropriate washing steps, biotin labeled polyclonal anti-rat fibrinogen primary antibody binds to the captured protein. Excess antibody is washed away and bound polyclonal antibody is reacted with avidin conjugated to horseradish peroxidase. TMB substrate is used for color development at 450nm. A standard calibration curve is prepared along with the samples to be measured using dilutions of rat fibrinogen. Color development is proportional to the concentration of fibrinogen in the samples.

MATERIALS PROVIDED

Component	Contents	Quantity	Storage	Cat. No.
Coated Plate	96-well plate coated with anti-rat fibrinogen	1 plate	4°C	FB07a
Standard	Rat fibrinogen standard (lyophilized)	1 vial	4°C	FB07b
Wash Buffer	10x solution for washing plate	50 mL	4°C	FB07c
Dilution Buffer	5x solution for diluting kit reagents and samples	50 mL	4°C	FB07d
Primary Antibody	Biotin labeled anti-rat fibrinogen (lyophilized)	1 vial	4°C	FB07e
Secondary Conjugate	Avidin-HRP enzyme conjugate	1 vial	4°C	FB07f
Substrate	TMB substrate	10 mL	4°C	FB07g
Stop Solution	1 M sulfuric acid to stop substrate reaction	6 mL	4°C	FB07h

MATERIALS NEEDED BUT NOT PROVIDED

1. DI Water
2. Microplate reader with 450 nm filter
3. Microplate shaker with uniform horizontal circular movement up to 300 rpm (optional)
4. Precision pipettes that range from 10 µL-1000 µL and disposable tips (single- and multi-channel)

STORAGE

1. Store the kit and all of its components at 4°C before use.
2. If not using the entire plate at once, prepare only the appropriate amount of Primary Antibody and Standard. The remaining stock solutions should be frozen and stored at –70°C. Do not freeze/thaw more than once. All other components should remain refrigerated.
3. Store unused portions of the microplate in a pouch with a desiccant at 4°C.

PROCEDURAL NOTES

1. This assay should be run at room temperature.
2. Use aseptic technique when opening and dispensing reagents.
3. This kit is designed to work properly as provided and instructed. Additions, deletions or substitutions to the procedure or reagents are not recommended, as they may be detrimental to the assay.
4. To minimize error due to handling, wipe the exterior bottom of the microplate wells with a lint-free paper towel.

SAMPLE COLLECTION, STORAGE, AND PREPARATION

Samples giving rat fibrinogen levels above 800 ng/ml should be diluted in 1X Dilution Buffer before use. Normal plasma samples need to be diluted between 1:10,000 and 1:50,000 in 1X Dilution Buffer for the values to be within the linear range of the standard curve.

REAGENT PREPARATION

1. **10x Wash Buffer:** Dilute the 50 mL of concentrate to 1x with 450 mL of DI water prior to use.
2. **5x Dilution Buffer:** Dilute the 50 mL of concentrate to 1x with 200 mL of DI water prior to use.
3. **Primary Antibody:** Reconstitute with 1x Dilution Buffer as directed on the vial and vortex gently to mix. Prepare immediately prior to use.
4. **Secondary Conjugate:** Dilute with 1x Dilution Buffer as directed on the vial and vortex gently to mix. Prepare immediately prior to use.

STANDARD PREPARATION

Reconstitute the standard by adding 5.0 mL of dilution buffer directly to the vial and agitate to fully dissolve. This will provide a concentration of 800 ng/mL solution. Prepare the serial dilution as outlined in the table below.

Standard	Fibrinogen Concentration (ng/mL)	Amount of 1x Dilution Buffer (µL)	Transfer Volume (µL)	Transfer Source	Total Volume (µL)
S_9	800	---	1000	Stock Vial	500
S_8	400	500	500	S_9	500
S_7	200	500	500	S_8	500
S_6	100	500	500	S_7	500
S_5	50	500	500	S_6	500
S_4	25	500	500	S_5	500
S_3	12.5	500	500	S_4	500
S_2	6.25	500	500	S_3	500
S_1	3.125	500	500	S_2	500
S_0	0	500	----	---	500

ASSAY PROCEDURE

NOTE: If a plate shaker is not available, increase the incubation time to 60 minutes for each step. The final absorbance values may be lower than if the assay was shaken.

1. Add 100 µL of standards or unknowns to each well. See **Scheme I** for a suggested plate layout. Shake the plate at 300 rpm on a plate shaker for 30 minutes.
2. Wash wells according to the following wash procedure:
   a. Remove contents of the plate by inversion into an appropriate disposal device.
   b. Tap out remaining contents of the plate onto a lint free paper towel.
   c. Add 300 µL of Wash Buffer to each well.
   d. Let stand for 2-3 minutes.
   e. Remove contents of the plate by inversion into an appropriate disposal device.
   f. Repeat procedure 2 more times and proceed to step "g".
   g. Tap out the remaining contents of the plate onto a lint free paper towel and proceed to step 6.
3. Add 100 µL of diluted Primary Antibody to each well. Shake plate at 300 rpm for 30 minutes.
4. Wash wells according to step 2.
5. Add 100 µL of the diluted Secondary Conjugate to each well. Shake plate at 300 rpm for 30 minutes.
6. Wash wells according to step 2.
7. Add 100 µL of TMB Substrate to each well and incubate for 15-30 minutes with shaking.
8. Add 50 µL of Stop Solution to each well to stop the reaction and read the plate at 450 nm.

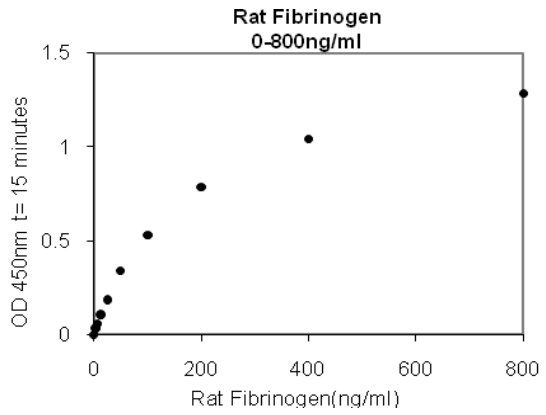
Scheme I:

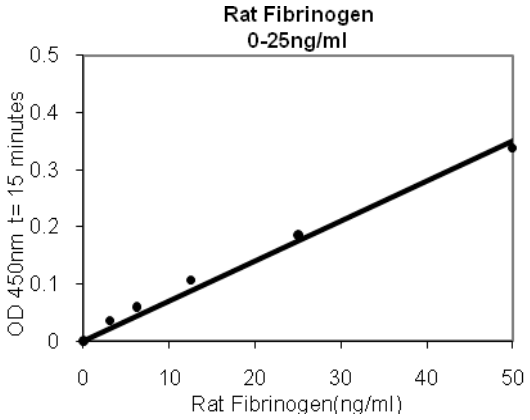
	1	2	3	4	5	6	7	8	9	10	11	12
A	S_0	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9	U_1	U_1
B	S_0	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9	U_1	U_1
C	U_3	U_4	U_5	U_6	U_7	U_8	U_9	U_{10}	U_{11}	U_{12}	U_{13}	U_{14}
D	U_3	U_4	U_5	U_6	U_7	U_8	U_9	U_{10}	U_{11}	U_{12}	U_{13}	U_{14}
E	U_{15}	U_{16}	U_{17}	U_{18}	U_{19}	U_{20}	U_{21}	U_{22}	U_{23}	U_{24}	U_{25}	U_{26}
F	U_{15}	U_{16}	U_{17}	U_{18}	U_{19}	U_{20}	U_{21}	U_{22}	U_{23}	U_{24}	U_{25}	U_{26}
G	U_{27}	U_{28}	U_{29}	U_{30}	U_{31}	U_{32}	U_{33}	U_{34}	U_{35}	U_{36}	U_{37}	U_{38}
H	U_{27}	U_{28}	U_{29}	U_{30}	U_{31}	U_{32}	U_{33}	U_{34}	U_{35}	U_{36}	U_{37}	U_{38}

CALCULATIONS

1. Average the O.D. values for each pair of duplicate wells.
2. Subtract the averaged O.D. of the zero point (S_0) from all other averaged values.
3. Plot a standard curve using the corrected O.D. versus the standard concentration.
4. Fit a straight line through the points using a linear fit procedure.
5. Determine the concentration of each unknown using the equation from the standard curve.

Typical Standard Curve:





EXPECTED VALUES

Fibrinogen is present in normal rat plasma at a concentration of 3.1 mg/ml[5] and varies by age and diet[6]. This assay measures total rat fibrinogen in the 3.125 - 800 ng/ml range.

REFERENCES

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4. Handa, K., *et al*.; (1989) Atherosclerosis **77:**209-213
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6. Dorner, H., *et al*., (1995) Gerontology **41:**252-259

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